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Activated oxygen production and detoxification in wheat plants subjected to a water deficit programme

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Abstract

Wheat plants (*Triticum durum* L. cv. Ofanto) were grown in a controlled environment. In one set, control plants were regularly watered; the other set of plants was subjected to two water deficit periods obtained by withholding water and rewatering to field capacity at the end of the first period. After both periods of stress, water potential (Ψ_w), pressure potential (Ψ_p) and relative water content (*RWC*) decreased; osmotic potential (Ψ_π) decreased by 0.3 MPa only after the second period of stress. In both treatments no osmoregulation mechanism occurred, however, an elastic adjustment took place and turgor was maintained at positive levels. Following the first treatment the good functionality of the ascorbate/glutathione cycle allowed the plants to maintain hydrogen peroxide to the control level despite a greater capacity of the thylakoid membranes to leak electrons towards oxygen; moreover, the ascorbate (AsA)/dehydroascorbate (DHA) ratio was unchanged while reduced glutathione (GSH)/oxidized glutathione (GSSG) increased in comparison with the control. Following the second period of stress, the decreased enzyme defence activities of the glutathione reductase (GR), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (AsAP) together with a minor glutathione content might be a consequence of a reduced rate of activated oxygen production.

Key words: Ascorbate/glutathione cycle, hydrogen peroxide, superoxide radical, *Triticum durum* L., water status.

Introduction

An organism which lives in an aerobic environment must constantly cope with the threat of oxidation and almost any cell process that involves oxygen can create activated oxygen.

Water stress conditions, in particular, may trigger an increased formation of the superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), which can directly attack membrane lipids and inactivate SH-containing enzymes (Navari-Izzo *et al.*, 1994). This formation is a consequence of the Mehler reaction, which provides a pathway for the removal of excess electrochemical energy determined by drought stress (Gamble and Burke, 1984). During water depletion $O_2^{\cdot-}$ can also react non-enzymatically with H_2O_2, giving rise to products such as hydroxyl radicals and singlet oxygen, which are even more reactive than $O_2^{\cdot-}$ itself (Elstner and Osswald, 1994; Navari-Izzo *et al.*, 1994).

Electron spin resonance (*ESR*) detection of free radicals in biological systems is normally not possible since the radicals are very unstable and terminate quickly by disproportionation or other mechanisms (McRae *et al.*, 1982). However, 1,2-dihydroxybenzene-3,5-disulphonic acid (Tiron), a disulphonated catechol that is readily oxidized to the corresponding semiquinone (a more stable free radical) by $O_2^{\cdot-}$, has been used to detect the superoxide radical in chloroplasts (McRae and Thompson, 1983; Miller and MacDowall, 1975; Navari-Izzo *et al.*, 1994; Sgherri *et al.*, 1993).

Photosynthetic cells can tolerate elevated oxygen levels because of endogenous mechanisms that effectively scavenge and remove the toxic products before cellular damage occurs (Halliwell, 1982).

In photosynthetically active cells, it is clear, however, that in the light, reductants generated as a consequence of electron transport can participate in the process of free radical scavenging, while in the dark or in heterotrophic tissue, various respiratory pathways must serve as com-

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parable sources of the reductants themselves (Alscher and Amthor, 1988).

Hydrogen peroxide, produced as a result of superoxide dismutase (SOD, EC 1.15.1.1) activity, is removed through the action of a metabolic cycle. This H_2O_2 detoxification cycle involves successive oxidations and re-reductions of glutathione, ascorbic acid and NAD(P)H (Gillham and Dodge, 1986).

The above-mentioned reactions are mediated by related enzymes such as ascorbate peroxidase (AsAP, EC 1.11.1.11), monodehydroascorbate reductase (EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and glutathione reductase (GR, EC 1.6.4.2). Rapid operation of the cycle is necessary since at least one of its constituent enzymes, AsAP, is inhibited by hydrogen peroxide itself (Hossain and Asada, 1984).

The purpose of this study was to detect the trend of H_2O_2 detoxification pathway constituents in plants subjected to a two-period stress programme. The aim is to gain a better understanding of the relation between water status of the plant and detoxification mechanisms in a drought-tolerant wheat (*Triticum durum* L. cv. Ofanto). The effects of water depletion on $O_2^{\cdot-}$ production and H_2O_2 content are also reported.

Materials and methods

Plant material

Seeds of a drought-tolerant wheat (*Triticum durum* L. cv. Ofanto) were provided by the Istituto Sperimentale per la Cerealicoltura (Foggia, Italy). The sensitivity to drought of the wheat plants had been determined by its grain yield during a five-year period in different areas of Southern Italy (Mariani and Novaro, 1993).

Wheat seedlings were grown at a day/night temperature regime of 20/15 °C with a 16 h photoperiod and a photon flux density of 400 μE m^{-2} s^{-1} and 80% R.H.

Twenty-nine-day-old plants were subjected to a two-period water deficit programme. Water was withheld for 12 d, then plants were rewatered to field capacity before another 12 d of water deficit. Control plants were maintained at field capacity by regular watering every 2 d at the same time of day. Plants from the control and the treatments were harvested at the end of each stress period. Roots and shoots were separated, the fresh weight was recorded, and samples were taken for dry weight measurements.

Leaf water status

The plants (three replicates of ten plants for each treatment) were collected at random early in the morning. Leaf water status was determined by pressure–volume curves using a pressure chamber. Curve analyses and the bulk modulus of elasticity (ϵ) were obtained according to Navari-Izzo *et al.* (1993).

Isolation of thylakoid membranes

Leaves (20 g) were homogenized with a Waring blender four times for 20 s each in an ice-cold chloroplast isolation medium (1:3, w/v) containing 0.5 M sucrose, 75 mM HEPES–KOH (pH 7.5), 10 mM diethyldithiocarbamic acid, 3.6 mM cysteine, and 5 mM EDTA. The homogenate was filtered through two layers of Miracloth and then centrifuged at 200 *g* for 5 min, and the supernatant centrifuged at 3000 *g* for 10 min. Thylakoid membranes were obtained as previously described by Sgherri *et al.* (1993). All steps were carried out at 4 °C.

ESR measurements

Electron Spin Resonance spectra were recorded at 25 °C using a Varian E-112 spectrometer equipped with a Varian variable temperature accessory. The spectrometer was interfaced to an AST Premium 486/25 EISA computer by means of an acquisition board (Ambrosetti and Ricci, 1991) and a software package designed for *ESR* measurements (Pinzino and Forte, 1992). The thylakoid preparations were diluted to 0.2 mg chl ml^{-1} with an oxygen-saturated-buffer consisting of 50 mM Tricine–KOH (pH 7.4), 0.33 M sucrose and 10 mM Tiron. The controls were (i) thylakoid preparations containing 0.35 mM ascorbate; (ii) thylakoid preparations containing 5 mM L-epinephrine; (iii) buffer solution containing 10 mM Tiron and 0.33 M sucrose without thylakoids. The reaction mixture was put into a 1 mm quartz sample tube sealed at one end and inserted into the microwave cavity of the spectrometer. The sample was then illuminated (600 W m^{-2}) with a 150 W tungsten lamp positioned 20 cm away from the cavity during the whole experiment. Kinetic measurements of $O_2^{\cdot-}$ production by illuminated thylakoids were determined by holding the magnetic field at a constant value and recording, as a function of time, the amplitude of the low-field spectral line of the Tiron semiquinone radical, which is formed when Tiron reacts with $O_2^{\cdot-}$. For the construction of kinetic curves, the amplitude of the first-line *ESR* spectrum was recorded every 20 s for a period of 15 min. The *ESR* parameters used were: microwave power, 10 mW; microwave frequency, 9.16 GHz; modulation amplitude, 1 G; gain and time constant, 0.064 s. The spectrometer conditions were selected so that the endogenous signals such as that of P_{700} or endogenous quinones did not interfere with the determination of changes in the Tiron semiquinone signal (Miller and MacDowall, 1975; Greenstock and Miller, 1975).

Hydrogen peroxide determination

Extraction and assay of H_2O_2 were performed as previously reported (Sgherri *et al.*, 1994*b*). A specific standard curve in the 0–15 nmol H_2O_2 range was used.

Ascorbate and dehydroascorbate determination

Leaf tissue was homogenized in 5% (w/v) trichloroacetic acid containing 4% (w/v) polyclar AT. Ascorbate (AsA) and total ascorbate (AsA + DHA) were determined in the supernatant as previously reported (Sgherri *et al.*, 1994*b*) and a specific standard curve covering the range 0–10 nmol AsA was used. Dehydroascorbate (DHA) levels were estimated on the basis of the differences between total ascorbate and AsA values.

Reduced and oxidized glutathione determination

Leaf tissue was homogenized in ice-cold 5% sulphosalicylic acid and the 5,5′-dithio-*bis*-nitrobenzoic acid (DTNB)-GR recycling procedure was used to determine total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) as previously described (Sgherri *et al.*, 1994*b*). Specific standard curves covering the range of 0–10 nmol reduced glutathione (GSH) and the range of 0.1–2 nmol for GSSG were used for total glutathione and GSSG determinations, respectively. GSH was

calculated by subtraction of GSSG as GSH equivalents from total glutathione.

Enzyme extractions and assays

All operations were carried out at 0–4 °C. Extractions and assays for GR, AsAP and DHAR were carried out following the procedure previously reported (Sgherri *et al.*, 1994*b*). For the AsAP assay, corrections for the low, non-enzymatic oxidation of AsA by H_2O_2 and for the oxidation of AsA in the absence of H_2O_2 were necessary. For the DHAR assay, controls without enzyme and without GSH were carried out to correct for non-enzymic reduction and for AsA present in the fraction being assayed, respectively. Proteins were determined according to Bensadoun and Weinstein (1976), using bovine serum albumin as a standard.

Results

After both periods of water stress the wheat plants showed a decrease of water potential (Ψ_w), pressure potential (Ψ_p) and *RWC* (Table 1). Osmotic potential (Ψ_π) did not change after the first period of stress, while it decreased by 0.3 MPa in stressed plants following the second period of stress. Osmotic potential at full turgor (Ψ_π^{100}) did not change after water depletion; however, ϵ decreased by 0.3 and 0.4 MPa following the first and second periods of stress, respectively (Table 1).

When Tiron was added to the sample and then illuminated, a four-line *ESR* spectrum was obtained (Sgherri *et al.*, 1993). The two hyperfine splitting constants for the Tiron semiquinone, ascribable to the interaction of the unpaired electrons spinning with the two non-equivalent ring protons, were 1.72 G and 3.23 G, respectively. Since Tiron reacts with $O_2^{\cdot-}$ to form the semiquinone radical, the amplitude of the Tiron *ESR* spectrum is proportional to the amount of $O_2^{\cdot-}$ being formed. Cessation of illumination resulted in a fall in the observed signal. When $O_2^{\cdot-}$ scavengers ascorbate and L-epinephrine were added to the sample preparations, and then illuminated, the Tiron radical signal was completely obscured (data not shown). The addition of ascorbate and L-epinephrine to the illuminated samples did not determine a reduction in the half-life of the signal on darkening, which indicated that these compounds react with superoxide rather than directly with the Tiron radical.

When the illumination was left on, the time-course of Tiron *ESR* signal amplitudes was as in Fig. 1, in which a three–phasic light-dependent production of the Tiron semiquinone was observed. A rapid signal increase, a steady-state phase and a signal decrease were originated by the simultaneous action of first order kinetics of formation and decay of Tiron semiquinone. The steady-state signal was reached over a period of about 3 min. This amplitude represents a condition where the rates of disproportionation of the radical and oxidation of the semiquinone by oxygen balance the rate of electron transfer from Tiron to superoxide anion. Thus, to evaluate the actual capacity of thylakoids to leak electrons towards oxygen, it was necessary to calculate the rate constant of first order kinetics of superoxide formation (K_f). The maximum amplitude (A_0) of the *ESR* signal in the absence of decay rate (K_d) represents the total superoxide production in our detection system. Indeed, the *ESR* signal reached this maximum value and then remained constant as soon as oxygen in the reaction mixture was completely consumed. The values reported in Table 2 were obtained by a non-linear best fitting of the experimental points shown in Fig. 1. The superoxide formation rate is $K_f = \ln 2\ (t_{1/2})^{-1}$, where $t_{1/2}$ is the time required to obtain the $A_0/2$ value.

$O_2^{\cdot-}$ production increased in stressed plants after the first period of stress, while it decreased after the second period (Fig. 1; Table 2). K_d value decreased after the first period of stress, while it remained unchanged after the second period (Table 2).

After both periods of stress H_2O_2 content remained at a constant level (Fig. 2) and total ascorbate content did not change, while AsA increase by *c.* 20% following the second stress period. According to this trend the AsA/DHA ratio remained unchanged after the first period

Table 1. *Effect of two periods of water deficit upon the water status and bulk modulus of elasticity in plants of* Triticum durum *L. cv. Ofanto*

Results are the means of three replicates of ten plants each. For comparisons among means the analysis of variance was used. For each period means in rows followed by different letters are significantly different at $P \leq 0.01$ level. ϵ, Bulk modulus of elasticity; Ψ_π, osmotic potential; Ψ_π^{100}, osmotic potential at full turgor; Ψ_p, pressure potential; Ψ_w, water potential; *RWC*, relative water content.

Parameter	First period		Second period	
	Control	Stress	Control	Stress
Ψ_w (MPa)	−0.5 a	−1.1 b	−0.6 a	−1.3 b
Ψ_π (MPa)	−1.5 a	−1.7 a	−1.3 a	−1.6 b
Ψ_p (MPa)	1.0 b	0.6 a	0.7 b	0.3 a
Ψ_π^{100} (MPa)	−1.0 a	−1.0 a	−1.0 a	−1.0 a
RWC (%)	92.5 b	76.9 a	92.5 b	77.3 a
ϵ (MPa)	3.6 b	3.3 a	2.6 b	2.2 a

Table 2. *Formation of decay of Tiron semiquinone in illuminated thylakoids of* Triticum durum *L.cv. Ofanto subjected to two periods of water depletion*

Results are means of three replicates of four separate experiments. For comparisons among means the analysis of variance was used. The significance of the letters is the same as in Table 1. K_f, formation rate constant; K_d, decay rate constant.

Parameter	First period		Second period	
	Control	Stress	Control	Stress
K_f (10^3 s^{-1})	18.5 a	21.3 b	23.1 b	16.3 a
K_d (10^6 s^{-1})	73.9 a	66.0 a	57.6 a	54.9 a

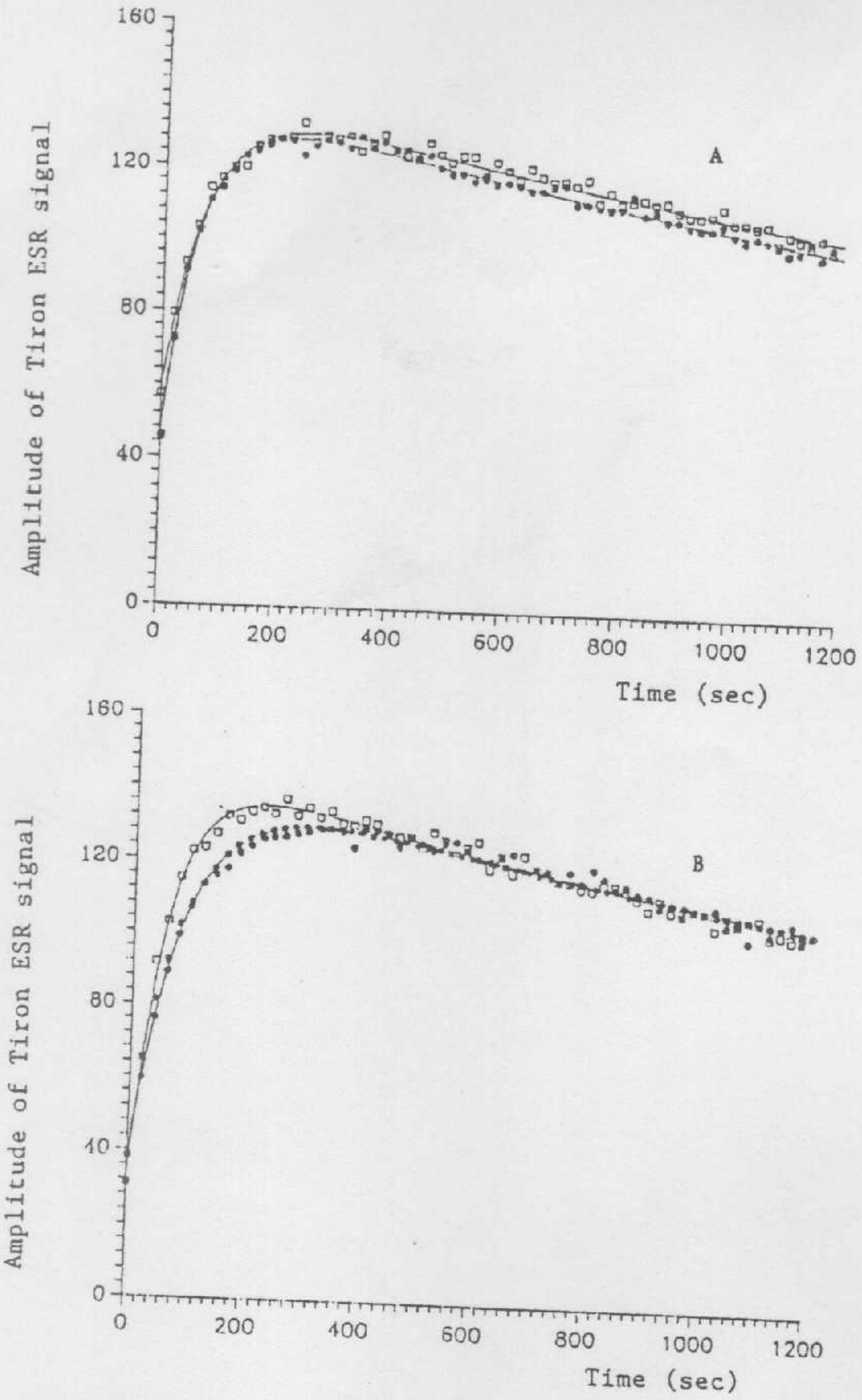

Fig. 1. Kinetic measurements of superoxide radical ($O_2^{\cdot-}$) production by illuminated thylakoids in plants of *Triticum durum* L. cv. Ofanto subjected to two periods of water stress. (A) First period; (B) second period; (□) control; (●) stressed; continuous lines, least-square-fit curves.

of stress, while at the end of the experiment in the stressed plants it was *c.* 2.5 times higher than in the control (Fig. 3).

Total glutathione and GSH contents showed a decrease in stressed plants after both treatments. The GSH/GSSG ratio had increased following the first period of stress, subsequently it had a 2-fold decrease in the stressed plants in comparison with the control (Fig. 4).

The enzyme activities of AsAP, DHAR and GR, did not change after the first treatment, while following the second period of stress they decreased by 40, 50 and 27% of the control value, respectively (Fig. 5).

Discussion

According to the definition of Hsiao (1973) the wheat plants reached a moderate level of water stress following

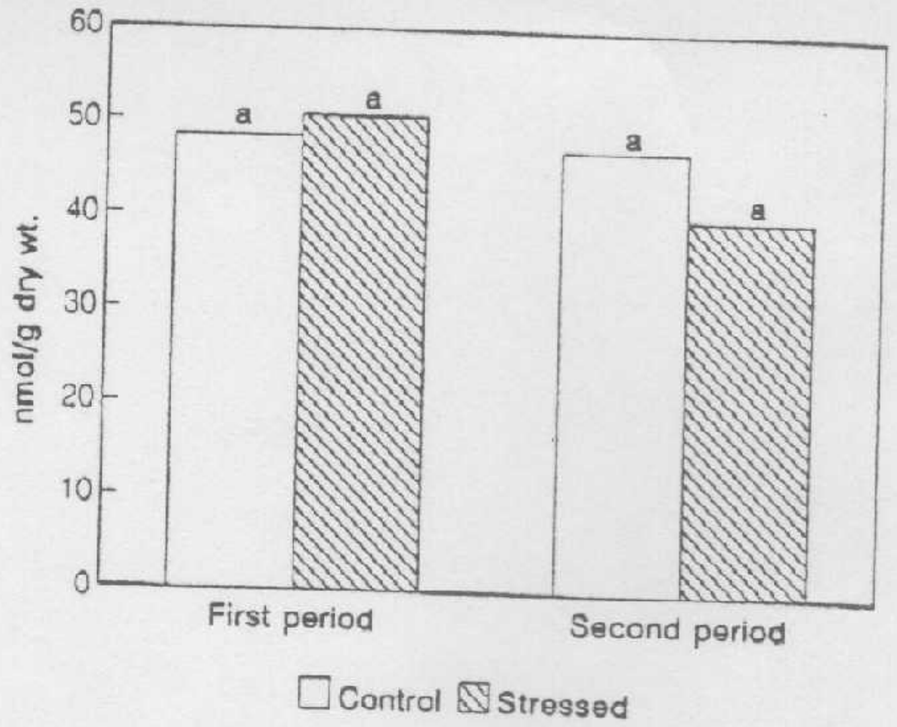

Fig. 2. Hydrogen peroxide content in plants of *Triticum durum* L. cv. Ofanto subjected to two periods of water stress. Results are the means of three replicates each analysed twice. For comparisons among means analysis of variance was used. For each period histograms accompanied by different letters are significantly different at $P \leqslant 0.01$.

both periods of stress (Table 1) and as is shown by the Ψ_π^{100} values no osmoregulation mechanism occurred. The maintenance of positive levels of turgor might be ascribed to the decreased bulk modulus of elasticity that took place after both periods of stress (Table 1). Indeed, a tissue with a small ϵ is an elastic one and it can sustain a smaller decrease in Ψ_p, for the loss of a given volume of water, than a more rigid tissue (Navari-Izzo *et al.*, 1993).

When thylakoids are illuminated, a light–oxygen-dependent formation of Tiron radical signal occurs, thus resulting in first order kinetics of formation and decay of *ESR* signal. Indeed, the rate of formation of $O_2^{\cdot-}$ in our systems is rate limiting (Greenstock and Miller, 1975; Miller and MacDowall, 1975) and under anaerobic conditions, i.e. helium or nitrogen saturation of the sample, no appreciable amount of Tiron signal could be induced by light. Furthermore, re–aeration of the sample restored the signal to the original magnitude (McRae *et al.*, 1982; McRae and Thompson, 1983; Miller and MacDowall, 1975; Miller and Rapp, 1973) and experiments on the influence of Tiron or benzoquinone on endogenous Mehler reactivity by wheat thylakoid preparations have clearly defined the stoichiometry of the reaction between Tiron and $O_2^{\cdot-}$ (Miller and MacDowall, 1975).

The dependence of Tiron radical signal generation on oxygen together with its being obscured by the $O_2^{\cdot-}$ scavengers ascorbate, L-epinephrine or reduced glutathione, confirms that it is derived from $O_2^{\cdot-}$ (Miller and MacDowall, 1975; Price *et al.*, 1989). L-epinephrine and ascorbate exhibited free accessibility to the site of oxygen reduction. On the other hand, added SOD may not always be effective in this role. It has been postulated that there is an apparent separation of the site producing superoxide from the enzyme, thus reflecting a steric problem in which the SOD is unable to access $O_2^{\cdot-}$ which

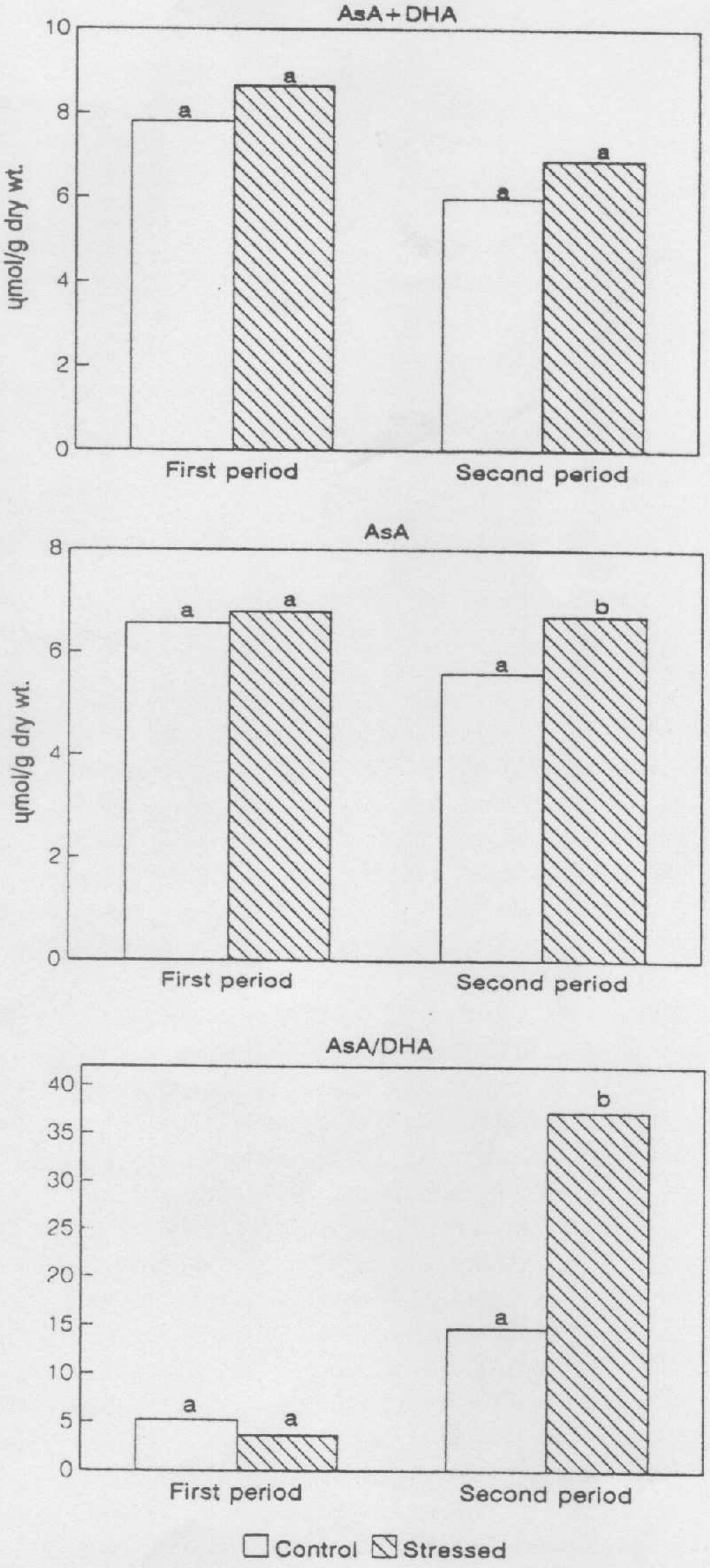

Fig. 3. Ascorbic acid content in plants of *Triticum durum* L. cv. Ofanto subjected to two periods of water deficit. The significance of the letters is the same as in Fig. 2. DHA, dehydroascorbate; AsA, reduced ascorbate.

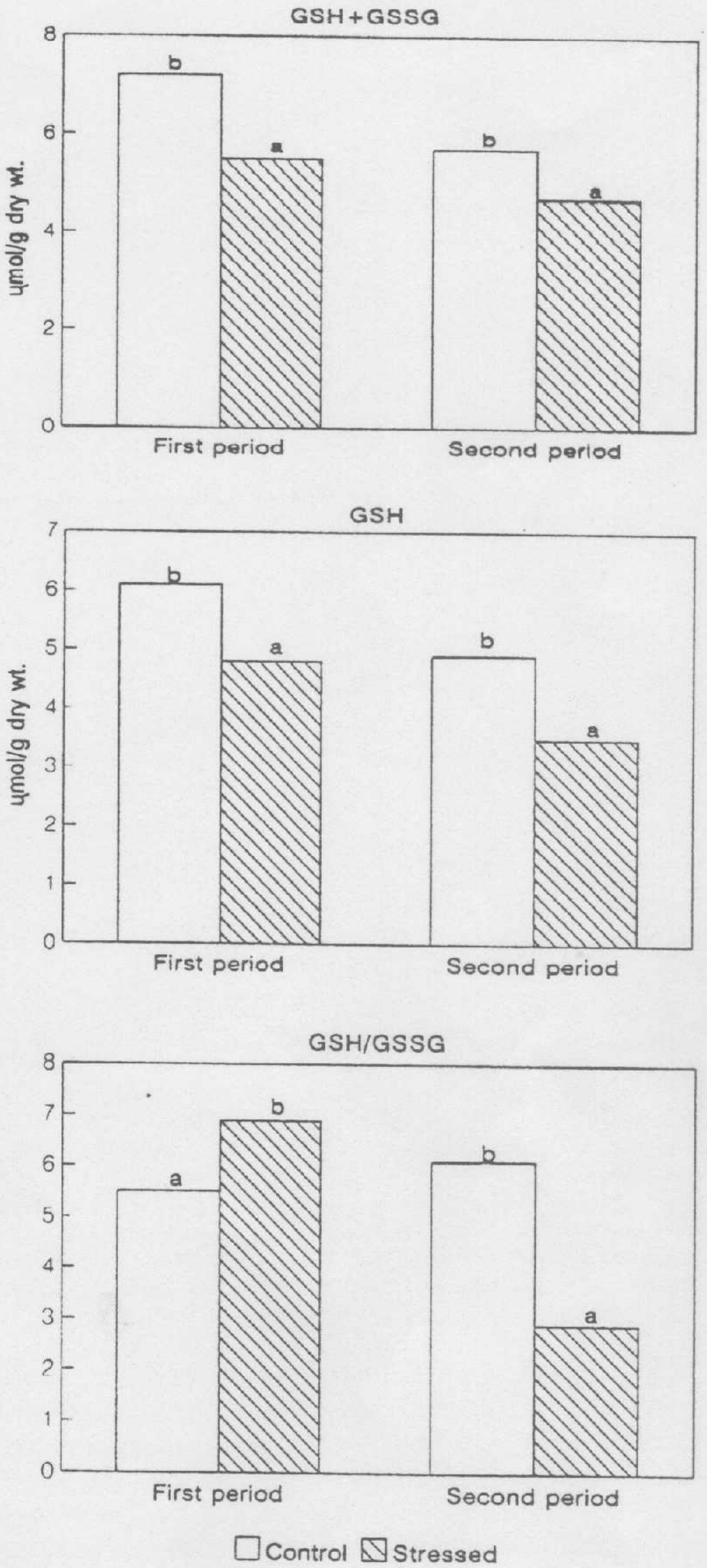

Fig. 4. Glutathione content in plants of *Triticum durum* L. cv. Ofanto subjected to two periods of water deficit. The significance of the letters is the same as in Fig. 2. GSSG, oxidized glutathione; GSH, reduced glutathione.

is available to the much smaller Tiron molecule (McRae and Thompson, 1983; Miller and MacDowall, 1975; Price *et al.*, 1989).

When oxygen became limiting in the sample, the Tiron radical decay rate overcame the Tiron radical formation rate and we observed a decline in the *ESR* kinetics (Fig. 1). Moreover, in thylakoid membranes of *Boea hygroscopica* where superoxide production, and presumably oxygen consumption, were very slow, the decay rate was about 23 d (Navari-Izzo *et al.*, 1994). If the sample

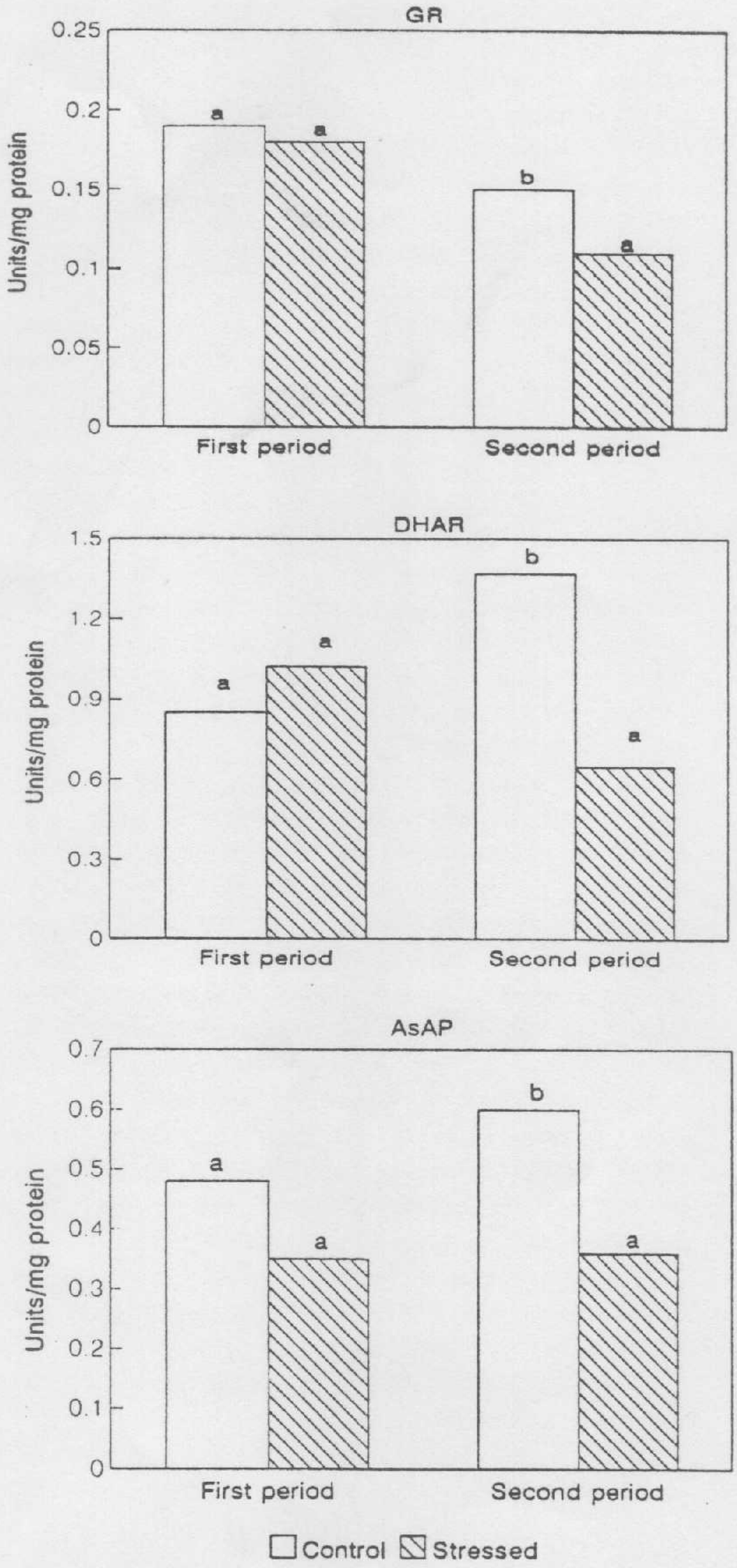

Fig. 5. Specific activities of the enzymes of the H_2O_2 detoxification cycle in plants of *Triticum durum* L. cv. Ofanto subjected to two periods of water depletion. The significance of the letters is the same as in Fig. 2. AsAP, ascorbic acid peroxidase; DHAR, dehydroascorbic acid reductase; GR, glutathione reductase.

could be saturated at ambient oxygen concentration for the whole time of the experiment, we may thus postulate a zero order kinetic of formation of superoxide. A first order process suggests an involvement of a reaction between Tiron semiquinone (limiting substrate) and other components of the reaction medium which were in excess (Miller and Rapp, 1973).

Production of superoxide by illuminated thylakoids was evaluated both by the calculation of the rate constant of superoxide formation, K_f, and by measuring steady-state amplitude when an equilibrium between rate of decay and rate of formation had been established. The use of K_f permits us to eliminate the problems associated with the decay of the radical, besides those linked to the use of the small sealed capillary in which the reaction mixture was enclosed, thus indicating the maximum capacity of thylakoids to leak electrons towards oxygen. This may reflect the structural properties of the membranes *per se*, i.e. a higher exposure of certain molecules to oxygen (McRae and Thompson, 1983; Sgherri *et al.*, 1993), rather than the total extent of Mehler activity, which can be underestimated using this system.

The evaluation of superoxide production by using the formation rate of superoxide without decay (K_f) may be an improvement in comparison with the past (McRae and Thompson, 1983; Miller and MacDowall, 1975; Miller and Rapp, 1973). Indeed, in the different samples the formation and decay rates of the *ESR* signal vary, so changing the time in which the steady-state is reached (Table 2).

Consistent with other observations of higher leakage rates of electrons to oxygen in thylakoids of plants subjected to dryland conditions (Sgherri *et al.*, unpublished), the thylakoid membranes show compositional and fluidity changes (Sgherri *et al.*, 1993; Quartacci *et al.*, 1995). After the first period of stress, the thylakoid membranes of the wheat plants also showed a slightly higher capacity to leak electrons towards oxygen (Table 2; Fig. 1) and, even if it might, in part, have contributed to increased H_2O_2 production, H_2O_2 accumulation did not occur (Fig. 2). Indeed, the enzyme activities of AsAP, DHAR and GR were maintained at the control levels together with the AsA/DHA ratio (Figs 3, 5) and the GSH/GSSG ratio increased (Fig. 4). According to Chowdhury and Choudhuri (1985), the H_2O_2 level can reliably be taken as an index for water stress tolerance. So, a good functionality of the ascorbate/glutathione cycle could explain the above result, indicating the capacity of this plant to withstand water depletion.

A high level of AsA is necessary for a plant's defence in water deficit conditions, since AsA, in addition to its role in the H_2O_2 detoxification cycle, can directly act as a scavenger of hydroxyl radicals (Halliwell and Gutteridge, 1985) and it can also function in the regeneration of alpha-tocopherol (Finckh and Kunert, 1985).

The importance of glutathione in the establishment of water stress tolerance has been pointed out (Sgherri *et al.*, 1994*a*, *b*). Different mechanisms seem to be involved in the revival of drought-tolerant plants. One of these is

evident in the ability of *Boea hygroscopica* to increase the small amount of the constitutive GSH up to 50 times following dehydration and then to utilize it (Sgherri *et al.*, 1994*a*). On the contrary, the maintenance of a low GSSG/GSH ratio, despite a decrease in total glutathione, is a mechanism involved in the drought tolerance of *Sporobolus stapfianus* (Sgherri *et al.*, 1994*b*). In wheat plants, subjected to a moderate level of water stress, the second of these trends might have been established in response to a decreased glutathione content (Fig. 4), as a consequence of its decreased synthesis and/or its increased degradation. Therefore, the greatest portion of the glutathione in the cell was maintained in the reduced state, playing an important role in the stabilization of many enzymes and a more general role as an oxidant scavenger, due to the fact that it serves as a substrate for DHAR (Wang *et al.*, 1991). GSH is also able to react directly with free radicals, including hydroxyl radicals, so preventing the inactivation of enzymes by oxidation of essential thiol groups (Wang *et al.*, 1991).

Under drought conditions $O_2^{\cdot-}$ production depends on species, intensity of stress, repeated stress periods and senescence. The steady-state amplitude of the Tiron radical signal of chloroplasts from 21-d-old bean leaves was about 4-fold that of chloroplasts isolated from 9-d-old leaves (McRae and Thompson, 1983). As a consequence, the only possible comparison is between the *ESR* data of the stressed sample and those of the control of the same age (Table 2).

The light-dependent O_2 reduction by thylakoids demonstrates a requirement for photosynthetic electron transport (Grace and Osmond, 1991; Sgherri *et al.*, 1993; Walker *et al.*, 1991). It is known that during the Mehler reaction molecular oxygen can serve as a NADPH-alternative acceptor of unpaired electrons, so as to create additional ATP and $O_2^{\cdot-}$ (Jennings and Forti, 1975). A close regulation of electron transport at low temperatures to prevent uncontrolled production of activated oxygen within the chloroplasts has been invoked by Walker *et al.* (1991) as a mechanism of chilling resistance in *Lycopersicon hirsutum*. This is supported by the observation that the electron transport rates are reduced in *L. hirsutum* exposed to chilling stress, whereas in the chilling-sensitive *L. esculentum* an increased electron transport was observed at low temperatures. Therefore, the lower $O_2^{\cdot-}$ production in the samples after the second period of stress in comparison with the control, might be related to the decreased thylakoid electron transport (Table 2; Fig. 1). A decreased efficiency of electron transport during water depletion might be an adaptive mechanism carried out by cv. Ofanto in order to limit oxidative damage. However, changes in $O_2^{\cdot-}$ production by thylakoids might not be strictly linked with the electron transport rate. The Tiron signal from intact chloroplasts was unaffected by 0.1 mM DCMU (McRae and Thompson, 1983) and, besides photosystem I sites, photosystem II is also a thermodynamically feasible site for electron leakage towards oxygen (Salin, 1987). Moreover, type I photodynamic reactions, undergoing charge separation within the excited pigment, have been postulated in the production of superoxide radicals (Elstner and Osswald, 1994), and a direct involvement of chlorophyll in the production of Tiron radical signal, via superoxide, has been previously demonstrated (McRae and Thompson, 1983). Following the second period of stress, the decline in superoxide production per unit of total chlorophyll (Fig. 1; Table 2) might also be due to a selective photobleaching of those chlorophyll molecules on the surface of the protein–chlorophyll complex and, therefore, more accessible to oxygen (McRae and Thompson, 1983).

Cellular maintenance, occurring as a consequence of free radical production, involves the reduction of harmful oxidative molecular species and the synthesis of molecules such as GSH. The processes involved in maintenance, when they include enzyme induction or antioxidant synthesis, have an energetic cost which has an effect on plant productivity (Alscher and Amthor, 1988).

A strict regulation of the activation of defence mechanisms under drought conditions could be important to avoid an excessive energy cost. Therefore, in wheat, following the second period of stress, the decreased enzyme activities involved in the H_2O_2 scavenging system, together with a lower glutathione content, might by a consequence of a more limited activated oxygen production (Figs 1, 2; Table 2). Total ascorbate content was less susceptible to change in comparison with glutathione (Figs 3, 4). The higher AsA/DHA ratio in comparison with the control, despite the decrease in DHAR, may be an indication of the key role, in the regenerating AsA, of the other ascorbate regenerating enzyme, monodehydroascorbate reductase (Arrigoni, 1994).

Thus, in wheat cv. Ofanto subjected to a second period of moderate water stress, there was a general rearrangement in the substrates and scavenging enzymes of the H_2O_2 detoxification cycle that might be related to the decreased oxidative threat.

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The nodulation of micro-propagated plants of *Parasponia andersonii* by tropical legume rhizobia

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Abstract

A simple clonal micro-propagation system for *Parasponia andersonii* was employed to study the nodulation response of this non-legume to inoculation by the broad host range *Rhizobium* sp. NGR234, isolated from *Lablab purpureus*, and also to tropical legume rhizobia isolated from *Aeschynomene* species. Partially effective nodules, assayed by acetylene reduction and ^{15}N dilution procedures, were induced with strain NGR234 and its spontaneous streptomycin-resistant mutant ANU240. Effective nodules were produced by one of the *Aeschynomene* strains (ORS302) tested, with rates of acetylene reduction comparable to those of root nodules produced by *Bradyrhizobium* strain CP279, originally isolated from *P. andersonii*. Light and transmission electron microscopy showed that there was a correlation between the nitrogen fixing capability of the symbiosis between NGR234 and *Parasponia* and the number of persistent infection (fixation) threads within the nodule cells.

Key words: *Parasponia*, *Bradyrhizobium*, *Rhizobium*, *Aeschynomene*, micro-propagation, root nodules, nitrogen fixation.

Introduction

The Gram-negative soil bacteria *Rhizobium* and *Bradyrhizobium* normally induce root nodules, which fix atmospheric nitrogen, on members of the family Leguminosae. It is now established that a group of 'slow-growing' rhizobia (bradyrhizobia) are also able effectively to nodulate species of the tropical non-legume genus *Parasponia* of the family Ulmaceae (Trinick, 1988; Trinick and Hadobas, 1988; Webster *et al.*, 1995). In contrast, fast-growing strains of rhizobia isolated from both temperate and tropical legumes have previously been shown either not to induce nodules on *Parasponia*, or to incite nodules on this plant that were partially effective or ineffective in nitrogen fixation (Trinick and Galbraith, 1980; Trinick and Hadobas, 1990; Becking, 1992). For example, previous experiments have demonstrated the formation of root nodules on *P. andersonii* with *Rhizobium* strain NGR234 (Trinick and Galbraith, 1980), a broad host range bacterium originally isolated from *Lablab purpureus* and known to nodulate more than 70 genera of legumes (Relić *et al.*, 1993). However, there is confusion regarding the effectiveness of nodules induced on the roots of *Parasponia* by NGR234 (Trinick and Galbraith, 1980; Bender *et al.*, 1988; Becking, 1992).

The development of a clonal micro-propagation system for *Parasponia andersonii* (Davey *et al.*, 1993; Webster *et al.*, 1993) has overcome the difficulties associated with the use of seed-derived material, such as restricted seed availability, poor germination and, subsequently, high seedling mortality (Bender and Rolfe, 1985; Becking, 1992). A reproducible micro-propagation system permits the rapid generation of large populations of plants, which can be inoculated with a range of rhizobia.

This paper reports the nodulation and nitrogen fixation capability of the symbiosis between tropical legume rhizobia and micro-propagated plants of *Parasponia andersonii* in the presence of different concentrations of fixed nitrogen. In particular, the nodulation of *P. andersonii* by *Rhizobium* strains isolated from *Lablab* and *Aeschynomene* species was compared with the nodulation of *P. andersonii* by *Bradyrhizobium* strain CP279, originally isolated from root nodules of *P. andersonii*. This in-

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